Crystallization and preliminary X-ray analysis of (R)-specific enoyl-CoA hydratase from *Aeromonas caviae* involved in polyhydroxyalkanoate biosynthesis

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Dimeric (R)-specific enoyl-coenzyme A (CoA) hydratase from *Aeromonas caviae* catalyzes the hydration of *trans*-2-enoyl-CoAs with carbon lengths of 4–6 to yield their corresponding (R)-3-hydroxyacyl-CoAs and is essential for polyhydroxyalkanoate (PHA) biosynthesis. The enzyme has been crystallized by vapour diffusion against a reservoir solution containing 20% polyethylene glycol 4000, 5% 2-propanol and 20 m*M* HEPES pH 7.0 at 298 K. Crystals belong to the monoclinic space group *C*2, with unit-cell parameters $a = 111.54\,(3)$, $b = 59.29\,(1)$, $c = 47.27\,(4)$ Å, $\beta = 113.04\,(2)°$ and contain a dimeric molecule in the asymmetric unit. Flash-cooling of a crystal at 100 K alters its unit-cell parameters to $a = 109.82\,(7)$, $b = 57.98\,(6)$, $c = 46.84\,(2)$ Å, $\beta = 112.71\,(3)°$. Native data to a resolution of 1.7 Å have been collected with 94.5% completeness and an R_{merge} of 4.0% under cryogenic (100 K) conditions using synchrotron radiation.

1. Introduction

Metabolism of various fatty acids *via* β-oxidation includes a hydration step in which 2-enoyl-CoA intermediates are converted to 3-hydroxyacyl-CoAs. Usually, (S)-specific enoyl-CoA hydratase [(S)-hydratase] is responsible for this reaction in various organisms. However, (R)-specific enoyl-CoA hydratase [(R)-hydratase] in peroxisomal multifunctional enzyme type 2 (MFE-2) is involved in the metabolism of straight-chain fatty acids in yeast and of very long chain or 2-methyl-branched fatty acids in mammals (Hiltunen *et al.*, 1992; Qin *et al.*, 1997; Dieuaide-Noubhani *et al.*, 1997). These two hydratases are thought to be evolutionarily unrelated on the basis of their lack of sequence similarity.

Recently, we have cloned and characterized a novel bacterial (R)-hydratase involved in the biosynthesis of an energy-storage material, polyhydroxyalkanoate (PHA), in *A. caviae* (Fukui & Doi, 1997; Fukui *et al.*, 1998). We have demonstrated that this enzyme is essential for the formation of (R)-3-hydroxyacyl-CoA monomers in *A. caviae* by linking between the β-oxidation and PHA synthesis pathways. Unlike the eukaryotic enzymes, this bacterial (R)-hydratase is a monofunctional enzyme forming a homodimer with a molecular weight of 27 908 Da. It is encoded as the *phaJ* gene in the PHA biosynthesis operon (*pha* operon), including the *phaC* and *phaP* genes coding for PHA synthase (Fukui & Doi, 1997) and granule-associated protein (unpublished results), respectively. It is specific for *trans*-2-enoyl-CoA β-oxidation intermediates with a short chain length of 4–6. Interestingly, the deduced amino-acid sequence showed significant homology with (R)-hydratase domains of the eukaryotic MFE-2s (*e.g.* 38.4% identity with the yeast enzyme for 73 amino-acid residues; Fukui & Doi, 1997), indicating that they are derived from a common ancestor. Very recently, cloning of other homologous bacterial enzymes from *Pseudomonas aeruginosa* (Tsuge *et al.*, 1999) and *Rhodospirillum rubrum* (Reiser *et al.*, 2000) have been reported.

It is an issue of interest whether the two hydratases with different stereoselectivity adopt a similar reaction mechanism. While the structure and the reaction mechanism of (S)-hydratase have been well studied (Engel *et al.*, 1996; Kiema *et al.*, 1999), this is not the case for (R)-hydratase (Qin *et al.*, 2000). In addition, as PHA is an attractive environmentally compatible material because of its biodegradability as well as its thermoplasticity (Doi, 1990), (R)-hydratase has potential for industrial application to produce engineered PHA and information on its structure–function relationship will be indispensable. To elucidate the reaction mechanism of (R)-hydratase on the basis of its structure, we have undertaken a crystallographic study of this enzyme. Here, we report the first crystallization and preliminary X-ray analysis of (R)-hydratase from *A. caviae*.

2. Materials and methods

2.1. Sample preparation

(R)-Hydratase from *A. caviae* was over-expressed in *Escherichia coli* BL21(DE3) cells harboring the expression plasmid pETNB3

crystallization papers

Data set	Native 1	Native 2
Wavelength (Å)	1.5418	1.0000
Temperature (K)	298	100
Resolution limit (Å)	2.5	1.7
Unit-cell parameters		
a (Å)	111.54 (3)	109.82 (7)
b (Å)	59.29 (1)	57.98 (6)
c (Å)	47.27 (4)	46.84 (2)
β (°)	113.04 (2)	112.71 (3)
Total observations	30245	51611
Unique reflections	9461	28412
Completeness (%)	93.0 (87.6)	94.5 (84.9)
$\langle I/\sigma(I)\rangle$	10.8	17.2
R_{merge}† (%)	7.2 (15.6)	4.0 (16.5)

† $R_{merge} = 100\sum|I_i - \langle I\rangle|/\sum I_i$, where I_i is the intensity of the ith observation, $\langle I\rangle$ is the mean value for that reflection and the summations are over all reflections.

containing the *phaJ* gene and purified by one-step anion-exchange column chromatography as described previously (Fukui *et al.*, 1998). The enzyme solution was concentrated to 28 mg ml^{-1} using Centriprep-10 (Amicon) and was subjected to an initial search for crystallization conditions. The homogeneity of the enzyme solution was judged by SDS–PAGE.

2.2. Crystallization

A sitting-drop vapour-diffusion technique was applied to crystallization of the enzyme using VDX plates and MicroBridges (Hampton Research). Each droplet was prepared on a MicroBridge by mixing 2.5 µl of the enzyme solution with an equal volume of reservoir solution and was equilibrated against 1 ml of the reservoir solution at 277 or 298 K. In initial screening with a sparse-matrix sampling approach (Jancarik & Kim, 1991), Crystal Screen solutions (Hampton Research) were used.
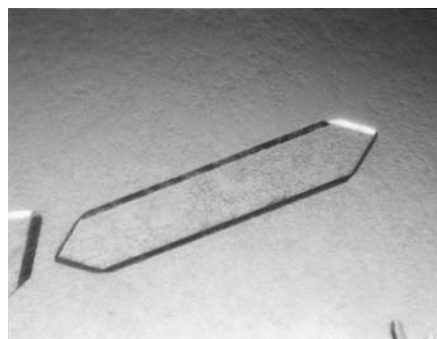
Figure 1
A crystal of (*R*)-specific enoyl-CoA hydratase from *A. caviae*. Its approximate dimensions are 0.5 × 0.1 × 0.04 mm.

2.3. X-ray experiments

A crystal was sealed in a thin-walled glass capillary tube with a small amount of reservoir solution. Cu $K\alpha$ X-rays were generated from a Rigaku UltraX18 rotating-anode generator operating at 45 kV and 90 mA, focused with a double-mirror system and monochromated with a Ni filter. Diffraction data were collected at room temperature using a Rigaku R-AXIS IV imaging-plate system. A total of 72 images were recorded with an oscillation angle of 2.5° and an exposure time of 20 min per image. The crystal-to-detector distance was set to 150 mm. Data were indexed using the data-processing software *PROCESS* (Rigaku).

High-resolution data were collected under cryogenic (100 K) conditions with synchrotron radiation (λ = 1.00 Å) at the BL44B2 station at SPring-8, Harima, Japan (Adachi *et al.*, 1996) using an R-AXIS IV imaging-plate detector. A total of 45 images were recorded with an oscillation angle of 2.0° and an exposure time of 5 min per image. The crystal-to-detector distance was set to 220 mm. Prior to data collection, a crystal was equilibrated in a stabilizing solution containing 28% polyethylene glycol 4000, 5% 2-propanol and 20 mM MES buffer pH 6.0, transferred to a cryoprotectant solution containing 10% glycerol, 25% polyethylene glycol 4000, 4.5% 2-propanol and 18 mM MES buffer pH 6.0 and then directly mounted on a CryoLoop (Hampton Research) and flash-cooled in a nitrogen stream at 100 K. The intensity data were processed with the program *DENZO* and scaled with the program *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

Crystals were obtained under several conditions with polyethylene glycol of various molecular weights (4000, 6000 and 8000) both at 277 and 298 K in the initial search for crystallization conditions using Crystal Screen. Because crystals were observed concomitant with amorphous precipitation of protein, subsequent crystallization experiments were carried out to optimize the conditions, including the protein concentration. Finally, using protein solution with a reduced concentration of 6 mg ml^{-1}, crystals with good morphology were grown from a reservoir solution containing 20% polyethylene glycol 4000, 5% 2-propanol and 20 mM HEPES buffer pH 7.0 at 298 K in two to three weeks (Fig. 1). However, they were not stable in

that reservoir solution when transferred for stock. Therefore, they were stabilized in a solution containing 28% polyethylene glycol 4000, 5% 2-propanol and 20 mM MES buffer pH 6.0. Adding 10% glycerol to the stabilizing solution was found to be effective against destabilization of crystals under cryogenic (100 K) conditions.

The crystals belonged to the monoclinic space group $C2$, with unit-cell parameters a = 111.54 (3), b = 59.29 (1), c = 47.27 (4) Å, β = 113.04 (2)°. Assuming two monomers in the asymmetric unit, the volume-to-mass ratio (V_M) was calculated to be 2.56 Å^3 Da^{-1}, which lies within the range often observed for protein crystals (Matthews, 1968). This value corresponded to a solvent content of about 52%. When the crystal was flash-cooled at 100 K, its unit-cell parameters changed slightly to a = 109.82 (7), b = 57.98 (6), c = 46.84 (2) Å, β = 112.71 (3)°.

Two native data sets were collected and the statistics of data collection are summarized in Table 1. At room temperature, a crystal diffracted Cu $K\alpha$ radiation to 2.0 Å resolution; diffraction data were collected and reduced to a resolution of 2.5 Å with a completeness of 93.0% and an R_{merge} of 7.2% (native 1). Calculation of the self-rotation function using the program *GLRF* (Tong, 1993) showed a signal (3.6σ) at φ = 135.5, ψ = 48.5 and κ = 180°, indicating that the dimeric molecule had non-crystallographic twofold symmetry. On the other hand, a flash-cooled crystal diffracted to 1.4 Å resolution with synchrotron radiation and diffraction data were collected and indexed to a resolution of 1.7 Å, with a completeness of 94.5% and an R_{merge} of 4.0% (native 2). Calculation of the self-rotation function showed no significant peaks. This might suggest that the non-crystallographic twofold rotation axis observed with the data collected at room temperature was altered in direction so as to be nearly parallel to the crystallographic twofold rotation axis, probably as a consequence of the rotation of molecules in the crystal which occurred upon flash-cooling.

A heavy-atom search has been attempted and three sets of derivative data have been collected at room temperature which give significant difference Patterson peaks. Structural analysis by multiple isomorphous replacement is now in progress.

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